Strategically Timing Inhibition of Phosphatidylinositol 3-Kinase to Maximize Therapeutic Index in Estrogen Receptor Alpha–Positive, PIK3CA-Mutant Breast Cancer

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Abstract

Purpose: Phosphatidylinositol 3-kinase (PI3K) inhibitors are being developed for the treatment of estrogen receptor α (ER)–positive breast cancer in combination with antiestrogens. Understanding the temporal response and pharmacodynamic effects of PI3K inhibition in ER+ breast cancer will provide a rationale for treatment scheduling to maximize therapeutic index.

Experimental Design: Antiestrogen-sensitive and antiestrogen-resistant ER+ human breast cancer cell lines and mice bearing PIK3CA-mutant xenografts were treated with the antiestrogen fulvestrant, the PI3K inhibitor GDC-0941 (pictilisib; varied doses/schedules that provided similar amounts of drug each week), or combinations. Cell viability, signaling pathway inhibition, proliferation, apoptosis, tumor volume, and GDC-0941 concentrations in plasma and tumors were temporally measured.

Results: Treatment with the combination of fulvestrant and GDC-0941, regardless of dose/schedule, was significantly more effective than that with single-agent treatments in fulvestrant-resistant tumors. Short-term, complete PI3K inhibition blocked cell growth in vitro more effectively than chronic, incomplete inhibition. Longer-term PI3K inhibition hypersensitized cells to growth factor signaling upon drug withdrawal. Different schedules of GDC-0941 elicited similar tumor responses. While weekly high-dose GDC-0941 with fulvestrant continuously suppressed PI3K signaling for 72 hours, inducing a bolus of apoptosis and inhibiting proliferation, PI3K reactivation upon GDC-0941 washout induced a proliferative burst. Fulvestrant with daily low-dose GDC-0941 metronomically suppressed PI3K for 6 to 9 hours/day, repeatedly inducing small amounts of apoptosis and temporarily inhibiting proliferation, followed by proliferative rebound compared with fulvestrant alone.


See related commentary by Toska and Baselga, p. 2099

Introduction

Approximately 70% of breast cancers express estrogen receptor α (ER) and/or progesterone receptor (PR), which typically indicate a degree of estrogen dependence. Patients with hormone receptor–positive breast cancer are treated with antiestrogen therapies [e.g., tamoxifen, fulvestrant (fulv), aromatase inhibitors (AI)] that inhibit ER signaling. While adjuvant therapy with antiestrogens prevents cancer recurrence, one third of patients develop antiestrogen-resistant advanced breast cancer that is rarely cured by approved therapies (1, 2).

We and others have shown that hyperactivation of the phosphatidylinositol 3-kinase (PI3K)/AKT/mechanistic target of the rapamycin (mTOR) pathway in preclinical models and human breast tumors promotes antiestrogen resistance (3, 4). PI3K is the most frequently aberrantly activated pathway in cancer, and alterations in genes encoding PI3K pathway proteins occur in >70% of ER+ breast cancers (data extracted from refs. 5 and 6). PI3K pathway activation promotes cell growth, proliferation, survival, and migration. Small molecule–mediated inhibition of PI3K, AKT, and/or mTOR suppresses antiestrogen-resistant growth of ER+ breast cancer cells and xenografts, particularly in models harboring gain-of-function mutations in PIK3CA (encodes the PI3K subunit p110α), the most commonly altered gene in ER+ breast cancer; ref. 5) or loss-of-function mutations in PTEN (encodes a phosphatase that antagonizes PI3K signaling...
Translational Relevance

One third of patients with estrogen receptor α (ER)–positive breast cancer develop antiestrogen-resistant disease that is virtually incurable. Activation of the phosphatidylinositol 3-kinase (PI3K) pathway promotes antiestrogen resistance. Combinations of antiestrogens and PI3K inhibitors are being developed; however, mechanistic understanding of the temporal effects is lacking, which is critical to inform rational treatment scheduling. Given that PI3K inhibitor treatment regimens being tested clinically provide continuous drug exposure, induce significant toxicity, and inconsistently provide robust pathway inhibition, we sought to determine whether antitumor efficacy could be achieved using pharmacodynamically informed treatment schedules. Different treatment schedules of a PI3K inhibitor elicited different patterns of pathway inhibition/reactivation, apoptosis, and proliferative inhibition/hyperactivation, while all eliciting therapeutic tumor responses. Clinical testing of different PI3K inhibitor treatment schedules is warranted to reduce toxicity while preserving efficacy. Furthermore, the concept of transient pathway interruption to increase therapeutic index may be extrapolated to the development of other signaling pathway modulators.

Materials and Methods

Cell lines

All parental cell lines were obtained from ATCC, cultured in DMEM/10% FBS (HyClone), and passaged for <3 months before analysis. Fulv-resistant (FR) MCF-7 (MCF-7/Fr) and T47D (T47D/Fr) cells were a gift from Matthew Ellis (Washington University in St. Louis, St. Louis, MO) and maintained in DMEM/10% FBS (HyClone) with 1 μmol/L fulv (Tocris Bioscience); cell lines were authenticated by mutational profiling using a 541-gene panel. ZR75-1/FR cells were generated by culturing ZR75-1 cells with 1 μmol/L fulv for 4 months. T47D/PI3KRB cells were a gift from Anthony Faber (Virginia Commonwealth University, Richmond, VA).

Sulforhodamine B (SRB) assay

Cells were seeded at 3,000–5,000/well in 96-well plates. The next day, cells were treated with 0 to 2 μmol/L GDC-0941 (kindly provided by Genentech) ± 1 μmol/L fulv for up to 5 days. Relative numbers of adherent cells were determined by SRB staining as previously described (26).

Xenograft studies

Animal studies were approved by the Dartmouth College IACUC. Female NOD-scid/IL2Rγ−/− (NSG; NOD.Cg-Prkdcscid Iil2rgtmWj/SzJ) mice (6–7 weeks old; obtained from the Norris Cotton Cancer Center Transgenic and Genetic Construct Shared Resource) were subcutaneously injected with 5 × 105 to 10 × 106 MCF-7 cells in Matrigel (BD Biosciences), or orthotopically implanted with ~8-mm3 fragments of serially transplanted HCl-003 patient–derived xenograft tissue (a gift from Alana Welm, University of Utah, Salt Lake City, UT; ref. 27) in the inguinal #4 mammary fat pad; mice were subcutaneously implanted on the same day with a 17b-estradiol pellet (0.72 mg, 60-day-release; Innovative Research of America). In mice subcutaneously injected with T47D/FR cells, subcutaneous administration of 5 mg/wk fulv was initiated on the same day (clinical formulation; provides ER inhibition in MCF-7 tumors for ≥8 days, data not shown; kindly provided by AstraZeneca). Mice bearing tumors 500 to 1,000 mm3 were randomized to treatment with vehicle, 5 mg/kg fulv once-weekly (QW), GDC-0941 (100 mg/kg QD × 5 d QW, 100 mg/kg BID × 3 d QW, or 800 mg/kg QW, p.o. in 100 μL 0.5% methylcellulose/0.2%Tween–80), and combinations. Tumor volumes were measured twice weekly using calipers (volume = length2 × width/2). Tumors were harvested and cut in pieces for snap-freezing or formalin fixation followed by paraffin embedding (FFPE).

Immunoblotting

Immunoblotting of protein extracts from cells and frozen tumor fragments was performed as previously described (28).
Strategic PI3K Inhibition for ER\(^+\) Breast Cancer

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GDC-0941 (\(\mu\)mol/L) | 0.125 | 0.25 | 0.5 | 1 | 2

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MCF-7/FR (+ 1 \(\mu\)mol/L Fulv) | ZR75-1/FR (+ 1 \(\mu\)mol/L Fulv)

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Relative levels of (phospho)proteins were measured by densitometry using ImageJ software.

Immunohistochemistry

Five-micron sections of FFPE tumor tissue were used for hematoxylin and eosin staining, IHC with antibodies against Ki67 (Biocare Medical) or geminin (Santa Cruz Biotechnology), and TUNEL (DeadEnd Colorimetric System; Promega). Proportions of positively stained cells were counted in 3 random microscopic fields (×400 magnification) in each specimen.

Pharmacokinetic and pharmacodynamic analyses

Mice were treated with 100 or 800 mg/kg of GDC-0941. Blood was collected by cardiac puncture at 0 to 72 hours into tubes containing EDTA (0.5 mol/L final concentration) as an anticoagulant, and centrifuged at 2,000 × g for 5 minutes at 4°C. Plasma was removed and stored at −80°C.

A separate group of mice bearing MCF-7 tumors was treated with fulv. Three days later, mice were treated with GDC-0941 (100 mg/kg, 800 mg/kg, or 100 mg/kg at 0 and 12 hours). Tumors were harvested at 0 to 72 hours and snap-frozen. GDC-0941 concentrations in plasma and tumors were determined as previously described (29). Pharmacokinetic and pharmacodynamic parameters were estimated by analyzing GDC-0941 concentration or effect versus time using WinNonlin software. Pharmacokinetic and pharmacodynamic data were analyzed using a noncompartmental model and the log-linear trapezoidal rule. For pharmacokinetic parameters, Tmax and Cmax were the observed time to maximum concentration, and maximum concentration, respectively. The following pharmacokinetic parameters were calculated: area under the curve from zero to infinity [AUC(0–∞)], terminal elimination half-life (T1/2), and mean residence time (MRT). For apoptosis and proliferation, maximum impact (Amax for apoptosis; Pmax for proliferation) and time to reach maximum impact (Tmax) were the observed values, and area under the curve from 0 to 144 hours [AUC(0–144)] was calculated.

Statistical analyses

In vitro cell growth, tumor IHC, and TUNEL data were analyzed by ANOVA followed by a Bonferroni multiple comparison-adjusted post hoc test between groups. To estimate proportion/regression of tumors, the following linear-mixed model was used:

\[
\log_{10}(\text{tumor volume}) = a + b \times t \quad (\text{refs. 30–32})
\]

The intercept of the linear model (a) for each treatment group in each experiment estimates the log_{10} (tumor volume) at time 0 (baseline), and the slope (b) estimates the rate of growth/reduction.

Additional methods are provided in Supplementary Information.

Results

Transient, complete inhibition of PI3K more effectively suppresses cell growth than continuous, partial PI3K inhibition

PI3K inhibitors exhibit little clinical efficacy as single agents (16–18), induce ER activation (Supplementary Fig. S1), and are being tested in combination with antiestrogens for the treatment of patients with ER+ breast cancer (14, 15). We first found that longer-term exposure to GDC-0941 induced PI3K hyperactivation (indicated by phospho-AKT levels) upon drug washout in MCF-7, ZR75-1, and T47D ER+/HER2– breast cancer cells (Fig. 1A). Similarly, longer-term exposure to GDC-0941 induced hypersensitivity to growth factor stimulation upon drug washout (Fig. 1B), suggesting that (i) longer-term PI3K inhibition induces increases in PI3K activators and (ii) that shorter-term PI3K inhibition might avoid such rebound effects upon drug washout. To determine the duration of PI3K inhibition required to induce anticaner effects in ER+ breast cancer, cells were treated with 0 to 2 μmol/L GDC-0941 alone or in combination with 1 μmol/L fulv for 0 to 120 hours, followed by GDC-0941 washout. Relative numbers of viable cells were measured after 120 hours. In PIK3CA-mutant (p110αE545K) MCF-7 cells, a 3- to 6-hour exposure to pharmacologically achievable concentrations of GDC-0941 (1–2 μmol/L; ref. 17) decreased cell viability ≥50% (Fig. 1C). Continuous exposure to 1 to 2 μmol/L GDC-0941 induced near-complete growth inhibition. In contrast, lower concentrations of GDC-0941 (≤0.25 μmol/L) required longer durations of exposure (≥24 hours) to appreciably decrease viability, reflecting a relationship between duration and magnitude of PI3K inhibition, and cell viability. Single-agent fulv effectively inhibited growth of MCF-7 cells (Supplementary Fig. S2). Thus, we used fulv-resistant MCF-7/FR cells to assess the effects of fulv and GDC-0941 in combination. Although it has been reported that MCF-7 cells resistant to fulv exhibit hyperactivation of insulin-like growth factor-1 receptor (IGF-1R), HER2, and epidermal growth factor receptor (EGFR), indicating general upregulation of growth-stimulatory pathways (4), MCF-7/FR dose/exposure responses to GDC-0941 were similar to those seen in parental MCF-7 cells (Fig. 1D). Analyses in PIK3CA-mutant (p110αE545K) MDA-MB-361 cells and PTEN-deficient ZR75-1 and ZR75-1/FR cells confirmed that more complete PI3K inhibition (1–2 μmol/L GDC-0941) with shorter exposure (24–48 hours) generally provides better growth inhibition than incomplete PI3K inhibition (0.25–0.5 μmol/L) with chronic exposure (≥72 hours) (Figs. 1C and D; Supplementary Fig. S2). We did not observe these relationships in PIK3CA-mutant (p110αE1047K) T47D cells (Supplementary Fig. S2), possibly due to a genetic deficiency in BCL2L11 that encodes the pro-apoptotic protein Bim (Supplementary Fig. S2 and ref. 34). Expression of exogenous Bim increased sensitivity to short-term treatment with 1 to 2 μmol/L GDC-0941 (Supplementary Fig. S2).

To monitor the kinetics of early apoptotic events, we used MCF-7 and ZR75-1 cells stably expressing a caspase-3/7–
activatable luciferase reporter (35). Cells were treated ± 1 μmol/L fulv for 2 days before exposure to 1 μmol/L GDC-0941 for 12, 24, or 36 hours. While combination drug treatment increased caspase-3/7 reporter activity compared with either agent alone, short- and longer-term exposures to fulv/GDC-0941 provided similar degrees of reporter activity in MCF-7 (12, 24, and 36 hours) andZR75-1 cells (12 and 24 hours; Supplementary Fig. S3). These data suggest that (i) transient, complete inhibition of PI3K more effectively suppresses cell growth than continuous, partial inhibition; (ii) short- and longer-term complete PI3K inhibition elicits similar degrees of growth inhibition and apoptosis; and (iii) longer-term PI3K inhibition primes cells for PI3K hyperactivation upon drug washout.

Pharmacokinetics of GDC-0941

We next tested the hypothesis that infrequent, pulsatile, high-dose (i.e., weekly) PI3K inhibition elicits greater antitumor effects than frequent, metronomic, low-dose (i.e., daily) PI3K inhibition in vivo. GDC-0941 pharmacokinetic analyses were performed with a fulv treatment backbone. To allow time for downstream effects of fulv-mediated inhibition of ER transcriptional activity (Supplementary Fig. S4), mice were treated with fulv 3 days prior to administration of GDC-0941. GDC-0941 plasma concentrations peaked after 15 to 30 minutes (Fig. 2A; Supplementary Table S1). With 100 mg/kg GDC-0941, plasma concentrations decreased to a plateau phase after 1 hour, were maintained for 8 hours (6.8–10.7 μmol/L), and decreased below the limit of detection within 24 hours. With 800 mg/kg GDC-0941, the plateau phase was maintained through 24 hours (7.9–15 μmol/L), and drug concentrations decreased below detectable limits within 72 hours. Because GDC-0941 plasma concentrations dropped sharply at 9 hours after low-dose treatment (Fig. 2A), a third dosing schedule was included (100 mg/kg at 0 and 12 hours) to provide near-continuous exposure with bi-daily dosing. Mice bearing MCF-7 xenografts were treated as above to assess tumor pharmacokinetics. Intratumor concentrations of GDC-0941 peaked at 9 hours with low-dose and high-dose treatments, and at 21 hours with two low-dose treatments. With low-dose GDC-0941, the drug was undetectable at 18 hours after a single dose, and after 36 hours with a second dose (Fig. 2B; Supplementary Table S1). With high-dose GDC-0941, intratumor drug concentrations declined between 9 and 48 hours, decreasing below the detectable limit by 72 hours. Pharmacokinetic parameter estimation revealed that high-dose GDC-0941 provided increased half-life (T1/2), exposure [AUC(0-inf)], and MRT compared with low-dose GDC-0941 (Fig. 2B).

Pharmacodynamic effects of PI3K inhibition on ER+ breast tumors

Mice bearing MCF-7 xenografts were treated as in Fig. 2. It has been reported that baseline PI3K activity (as measured by P-AKT levels) and PI3K inhibitor-induced mTORC1 inhibition (as measured by decreased levels of P-S6) correlate with clinical response (36). Thus, we measured levels of P-AKT and P-S6 as markers of PI3K and mTORC1 activities, respectively. P-AKT and P-S6 levels were maximally suppressed after ~1 hour of both low- and high-dose GDC-0941 treatments and returned to baseline within 9 and 72 hours, respectively (Fig. 3A), while total AKT levels remained unchanged (data not shown). P-AKT and P-S6 levels were inversely correlated with intratumor concentrations of GDC-0941 (Supplementary Fig. S5). In agreement with in vitro findings (Supplementary Fig. S1), the combination of fulv and GDC-0941 increased tumor cell apoptosis. PARP cleavage (marker of apoptosis) occurred within 1 and 3 hours of high-dose and low-dose GDC-0941 treatments, respectively, and cleavage increased over time (Fig. 3A).

A second low-dose GDC-0941 treatment after 12 hours provided continued inhibition of PI3K and mTORC1 for ≥21 hours, and more PARP cleavage than a single low-dose treatment (Fig. 3B), suggesting that bi-daily low-dose treatment is sufficient to nearly continuously inhibit PI3K. As observed in vitro (Fig. 1A and B), markers of PI3K and mTORC1 activities rebounded above baseline after GDC-0941 washout (Fig. 3C; Supplementary Fig. S6). For example, while PI3K inhibition decreased P-S6 levels at early time points, P-S6 increased above baseline within 1 day after daily or bi-daily low-dose treatments, and 3 days after a single high-dose treatment (Supplementary Fig. S6). In sum, low-dose

Figure 2.
Pharmacokinetic analysis of GDC-0941. A, mice were treated with GDC-0941. Blood was collected at the indicated time points after drug administration, and plasma was used to measure GDC-0941 levels. B, mice bearing MCF-7 tumors were pretreated with fulv, and then treated 3 days later with GDC-0941.
and high-dose GDC-0941 inhibit PI3K/mTORC1 signaling for 6 to 9 and 72 hours, respectively, so bi-daily low-dose treatment should provide near-continuous pathway inhibition. However, drug washout permits pathway hyperactivation.

Longer-term PI3K inhibition increases apoptosis and suppresses proliferation in combination with antiestrogen therapy in MCF-7 tumors

Tumors harvested from mice at 24, 36, and 48 hours following 1 day of treatment with GDC-0941 (± fulv pretreatment) were analyzed by TUNEL to monitor apoptosis. Single agents induced minimal apoptosis (Fig. 4A; Supplementary Fig. S7). In combination with fulv, one or two (0 and 12 hours) low doses of GDC-0941 induced peak apoptosis in 6.7% and 15.9% of tumor cells at 24 hours, respectively; apoptotic cell numbers declined thereafter. In contrast, high-dose GDC-0941 induced peak apoptosis at 48 hours (30.1%); it is unclear whether this marked apoptosis is partially attributable to a lack of clearance of dead cells. Interestingly, apoptotic timing was associated with the presence of drug (compare Fig. 2B; Fig. 4A).

Ki67 has been widely used as a marker of proliferation, but non-mitotically active cells can express Ki67 in G1 phase. Thus, we also evaluated geminin positivity as a marker of cells in S–G2 phases, and geminin/Ki67 ratio indicates the proportion of proliferating cells in S–G2 (37). One or two low doses (0 and 12 hours) of single-agent GDC-0941 significantly decreased tumor cell proliferation as measured by the geminin/Ki67 ratio at 24 hours (52.1% decreased to 29.1% and 31.6%, respectively), which rebounded by 48 hours (37.7% and 36.4%; Fig. 4B). High-dose GDC-0941 similarly blocked proliferation as a single agent, with continued inhibition through 48 hours (52.1% to 19.3%). Single-agent fulv suppressed proliferation (34.9%), and combination treatment
Strategic PI3K Inhibition for ER\(^+\) Breast Cancer

Intermittent, longer-term PI3K inhibition is as effective as metronomic PI3K inhibition in combination with antiestrogen therapy against ER\(^+\)/PIK3CA-mutant breast tumors

While we previously found that smaller MCF-7 tumors regress in response to fulv (38), larger tumors (500–1,000 mm\(^3\)) are generally unresponsive (Supplementary Fig. S10 and Supplementary Table S2). GDC-0941 was administered at doses/schedules that provided similar total amounts of drug (Fig. 5A). Different doses/schedules of single-agent GDC-0941 only slowed tumor growth without notable loss of body weight. Surprisingly, the combination of fulv and GDC-0941, regardless of dose/schedule, induced near-complete tumor regression in most cases (Fig. 5B; Supplementary Fig. S10, and Supplementary Table S2).

To validate observations in MCF-7 tumors, mice bearing PIK3CA-mutant (p110\(^{H1047R}\), confirmed by DNA sequencing) T47D/HER2\(^+\) xenografts treated with fulv from the time of cell implantation were randomized to different GDC-0941 doses/schedules. All GDC-0941 co-treatments induced tumor regression or stasis through 4.5 weeks until daily low-dose GDC-0941–treated mice resumed tumor growth (Fig. 5C; Supplementary Fig. S11, Supplementary Fig. S12A, and Supplementary Table S2). Withdrawal of fulv at 6.5 weeks was associated with outgrowth of most tumors despite continued GDC-0941 treatment (Supplementary Fig. S11), suggesting that combined targeting of PI3K and ER is required for disease control.

We then tested these treatments in mice bearing estrogen-dependent, ER\(^+\)/HER2\(^-\)/PIK3CA-mutant (p110\(^{H1047R}\), HCl-003 patient–derived breast cancer xenografts. While fulv did not affect tumor growth, combined treatment with fulv and GDC-0941 (daily low-dose or weekly high-dose) induced tumor stasis (Fig. 5D; Supplementary Fig. S12, Supplementary Fig. S13, and Supplementary Table S2). Notably, tumors from combination-treated mice showed large areas of central necrosis (Supplementary Fig. S12), suggesting that antitumor effects were more significant than physically measurable.

**Intermittent, longer-term inhibition of PI3K induces a wave of apoptosis and proliferative inhibition, with proliferative rebound after recovery of PI3K activity despite continued ER inhibition**

Different doses/schedules of PI3K inhibition similarly induced tumor responses in combination with fulv (Fig. 5). However, pharmacodynamic data indicated that high-dose GDC-0941 more effectively increased apoptosis and suppressed proliferation compared with low-dose GDC-0941 (Fig. 4). Thus, we temporally profiled MCF-7 tumors from mice treated for up to 7 days. A single treatment of fulv plus high-dose GDC-0941 induced peak apoptosis at 48 hours (30.1%), which dropped to baseline (2.7%) by 72 hours when GDC-0941 was cleared, and below baseline thereafter (compare Fig. 2B; Fig. 6A). Fulv plus bi-daily low-dose GDC-0941 (3 days on, 4 days off) induced peak apoptosis at

with GDC-0941/fulv further decreased geminin/Ki67 ratio at 24 hours. Evaluation of geminin and Ki67 as individual markers showed similar patterns of drug effects (Supplementary Fig. S8 and Supplementary Fig. S9).

When GDC-0941 was cleared from tumors of mice co-treated with fulv, tumor cell proliferation rebounded above levels observed in tumors from fulv-treated mice (compare Fig. 2B; Fig. 4B); while we considered that this indicates synchronized S-phase entry of G1-arrested cells, further analyses (described below) indicated that these are likely genuine increases in proliferation. In contrast, fulv plus high-dose GDC-0941 suppressed proliferation through 48 hours. Taken together, these data suggest that (i) temporal changes in tumor cell apoptosis and proliferative rebound can be predicted based on GDC-0941 pharmacokinetics, (ii) fulv administered with daily low-dose GDC-0941 should continually promote G1 arrest and a modest degree of apoptosis, (iii) fulv plus bi-daily low-dose GDC-0941 should further increase apoptosis, and (iv) fulv plus weekly high-dose GDC-0941 should drastically suppress proliferation and induce apoptosis. However, evaluation of the longer-term effects of these treatments, particularly following GDC-0941 washout, requires a longer time course (below).

**Figure 4.**

Intermittent, longer-term PI3K inhibition increases tumor cell apoptosis and suppresses proliferation in combination with anti-estrogen therapy. Mice were pretreated with fulv 3 days before treatment with one or two doses of GDC-0941 as indicated. Tumors were harvested at 0–48 hours after GDC-0941 treatment, and analyzed by (A) TUNEL to assess apoptosis, or (B) IHC for geminin and Ki67 to assess cell proliferation. Geminin:Ki67 ratios were calculated. Data, mean of triplicate tumors ± SD.

*P* ≤ 0.05 by Bonferroni post hoc test as indicated with brackets.
24 hours (18.3%), and maintained apoptosis at ~10% until 72 hours when GDC-0941 was cleared (compare Fig. 2B and Fig. 6A). Fulv plus daily low-dose GDC-0941 induced 8% to 10% apoptosis that was maintained for 72 hours; despite continued metronomic PI3K inhibition (Supplementary Fig. S6), apoptosis levels returned to baseline thereafter (Fig. 6A). These findings suggest that the bulk of apoptosis occurs within the first 3 days following initiation of PI3K inhibitor therapy. Pharmacodynamic modeling of apoptosis rate over the course of 7 days [AUC_{0-144}] revealed that a single high dose of GDC-0941 with fulv cumulatively induces more apoptosis that daily or bi-daily low-dose GDC-0941 regimens with fulv (Fig. 6A).

A single treatment with fulv plus high-dose GDC-0941 decreased tumor cell proliferation by 48 hours (34.9% decreased to 11.4%), which rebounded above baseline following GDC-0941 washout (Fig. 6B, Fig. 2B; Supplementary Fig. S14). While it is conceivable that this washout-induced increase in the geminin/Ki67 ratio reflects synchronized S-phase entry of G1-arrested cells, increased geminin/Ki67 ratio persisted for up to 7 days, suggesting that PI3K reactivation promotes proliferation. Fulv plus low-dose GDC-0941 (daily or bi-daily) modestly decreased proliferation, but the geminin/Ki67 ratio increased above baseline after 2 to 3 days of treatment (Fig. 6B, Fig. 2B; Supplementary Fig. S14). AUC_{0-144} generated from pharmacodynamic modeling showed that fulv plus daily low-dose GDC-0941 cumulatively inhibited tumor cell proliferation modestly better than bi-daily low-dose GDC-0941/fulv or a single high dose of GDC-0941/fulv within a 1-week period (Fig. 6B). These results collectively indicate that

Figure 5.
Intermittent, longer-term PI3K inhibition is as effective as metronomic inhibition in combination with antiestrogen therapy for ER\(^+\) breast cancer. Mice (n = 7-9/group) bearing (B) MCF-7 tumors, (C) T47D/FR tumors, or (D) HCI-003 patient-derived xenografts were treated as indicated in A. In A, mice were treated with fulv starting at the time of xenografting, and fulv was stopped after 6.5 weeks of GDC-0941/fulv treatment. Mean \pm SEM is shown. *, \(P < 0.05\) compared with the vehicle-treated group, or as indicated with brackets. #, \(P < 0.05\) compared with the fulvestrant-treated group. &, \(P < 0.05\) compared with the GDC-0941-treated group at the same dose/schedule. Statistical analysis of B included data up to 6.5 weeks.
different schedules of PI3K inhibition with antiestrogen therapy provide similar antitumor efficacy due to cumulative effects on apoptosis and proliferation: (i) intermittent, longer-term PI3K inhibition (weekly high-dose GDC-0941) induces a burst of apoptosis and proliferative inhibition that rebounds above baseline upon PI3K reactivation; (ii) metronomic PI3K inhibition (daily low-dose GDC-0941) induces smaller amounts of apoptosis and proliferative inhibition with less proliferative rebound.

Discussion

Herein, we demonstrate that both metronomic (daily) and intermittent (weekly) inhibition of PI3K in combination with an antiestrogen induce regression of antiestrogen-resistant, ER⁺/PIK3CA-mutant breast tumors. Combined targeting of ER and PI3K was significantly more effective than single-agent treatments. Detailed pharmacodynamic and pharmacokinetic profiling revealed that the majority of tumor cell apoptosis occurred early during the course of treatment, temporally correlating with PI3K inhibition. In vitro analyses showed that both short- and long-term inhibition of PI3K effectively reduced cell viability. These findings support the clinical exploration of different treatment schedules of PI3K inhibitors that may increase the therapeutic index.

Kinase inhibitors are often developed with pharmacokinetic properties and/or clinical dosing schedules that provide continuous drug exposure and target inhibition. The theory underlying this “occupancy-driven pharmacology” is that longer occupation of an enzyme active site by the drug will translate into increased clinical efficacy. While drug target inhibition is often confirmed in blood, skin, or tumor biopsies in early-phase clinical studies, temporal analysis of molecular effects in tumors is rarely feasible. Thus, a mechanistic rationale for dosing schedules is often lacking. Given that PI3K inhibitor treatment regimens being tested clinically induce significant toxicity and inconsistently provide robust pathway inhibition (14–18, 36), we sought to determine whether antitumor efficacy could be achieved using alternate, pharmacodynamically informed treatment schedules. Indeed, weekly treatment with a high dose of GDC-0941 (800 mg/kg), daily treatment with a lower dose (100 mg/kg), or bi-daily treatment with a lower dose for 3 consecutive days per week (100 mg/kg BID × 3 days QW) all induced similar tumor responses when combined with fulvic in 3 models of ER⁺ breast cancer (Fig. 5; Supplementary Figs. S10, S11, and S13; Supplementary Table S2). Weekly high-dose and bi-daily low-dose treatments provided continuous PI3K inhibition for 48 to 60 hours (Fig. 3; Supplementary Fig. S6), while daily low-dose treatment metronomically inhibited PI3K for 6 to 9 hours/day. In correlation, intermittent (weekly and bi-daily) PI3K inhibition induced a bolus of apoptosis followed by a rebound in tumor cell proliferation, while metronomic (daily) PI3K inhibition repeatedly induced small amounts of apoptosis (Figs. 4A, 6A).
Genes encoding PI3K activators (e.g., IGF-1R, HER3) are transcriptionally upregulated in response to long-term (>4 hours), but not short-term (<2 hours), PI3K inhibition via activation of FoxO transcription factors (39). Long-term PI3K inhibition also upregulates ER levels and activity (Supplementary Fig. S1A), and ER promotes IGF1R transcription. Thus, the hyperproliferative tumor response to PI3K reactivation in mice treated with high-dose GDC-0941 (Fig. 6B; Supplementary Fig. S14) may be related to the hypersensitization of PI3K to growth factor stimulation following prolonged PI3K inhibition (Fig. 1A and B). We conclude that the sum of antiproliferative and pro-apoptotic effects of these treatment regimens is similar, providing comparable tumor responses. Whether the PI3K reactivation–associated hyperproliferative tumor response promotes an aggressive tumor phenotype that may affect disease progression requires further detailed study.

We found that MCF-7 tumors regressed to near completion with combined inhibition of ER and PI3K, while T47D/FR and HCl-003 tumors either slightly regressed or growth arrested (Fig. 5; Supplementary Figs. S10, S11, and S13). While inhibitors of oncogenic effectors kinases (e.g., PI3K, MEK) frequently inhibit cancer cell proliferation, apoptosis is required for regression of solid tumors (34). Hata and colleagues recently reported that the ratio of pro-apoptotic (e.g., Bim) to anti-apoptotic (e.g., Bcl-2, Bcl-xL) proteins dictates cell fate in response to PI3K and MEK inhibitors (40). We found that (i) MCF-7 tumors express a higher ratio of Bim:Bcl-2:Bcl-xL than T47D/FR or HCl-003 tumors, potentially "priming" MCF-7 tumors for apoptosis (41); (ii) T47D/FR tumors are Bim deficient; and (iii) HCl-003 tumors express high levels of Bcl-xL (Supplementary Figs. S1 and S15). Therefore, T47D/FR and HCl-003 tumors are predicted to regress if cotreated with a drug that sufficiently increases the balance of pro-apoptotic/anti-apoptotic proteins (e.g., the Bcl-2:Bcl-xL inhibitor navitoclax). These data also suggest that the nature of clinical response (regression versus stable disease) to ER/PI3K inhibition may be predictable based on the ratio of pro-apoptotic/anti-apoptotic proteins.

In ER+ breast cancer models, phosphorylation of ribosomal protein S6 is often controlled by a PI3K signaling cascade, placing mTORC1 and cap-dependent translation under PI3K control. Elkabets and colleagues reported that treatment-induced decreases in P-S6 levels are predictive of response to PI3K/p110α inhibition in PIK3CA-mutant breast cancer cells and human tumors, and mTORC1 reactivation is associated with drug resistance (36). While fulv/GDC-0941 initially decreased P-S6 levels in tumor models tested herein, P-S6 rebounded independently of P-AKT in some cases (Fig. 3; Supplementary Fig. S6). These findings suggest that (i) mTORC1 activation quickly becomes disconnected from PI3K/AKT due to signaling reprogramming and/or clonal selection, and (ii) using P-S6 as a pharmacodynamic biomarker to predict clinical response may be complicated by timing of assessment and compensatory mTORC1-activating pathways. Furthermore, therapeutic target-
Strategically Timing Inhibition of Phosphatidylinositol 3-Kinase to Maximize Therapeutic Index in Estrogen Receptor Alpha–Positive, PIK3CA-Mutant Breast Cancer

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*Clin Cancer Res* Published OnlineFirst January 5, 2016.